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Autologous Antibody Response against the Principal Neutralizing Domain of Human Immunodeficiency Virus Type 1 Isolated from Infected Humans

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High titers of neutralizing antibodies in human immunodeficiency virus type 1 (HIV-1) infection are directed primarily against the third hypervariable domain (V3) of the virion envelope glycoprotein gp120. This region has been designated the principal neutralizing domain of HIV-1. Because the frequency and significance of autologous V3 antibodies in natural infection are not fully clarified, we have cloned, sequenced, and expressed the V3 domain from virus of HIV-1-infected patients to test the autologous and heterologous V3 antibody response. The resulting recombinant *Escherichia coli* V3 fusion proteins reacted strongly with both autologous and heterologous patient antibodies in Western blots. Thirty-one different V3 fragments were cloned from 24 hemophiliac patients with different immunological and clinical statuses. Antibody reactivity against the autologous V3 fusion proteins was detected in all serum samples except one; moreover, all serum samples contained antibody reactivity against a vast majority of heterologous fusion proteins despite significant amino acid variability in V3. The results suggest that V3 antibodies are highly prevalent; further, we find no association between the stage of the HIV-1 infection and the presence of V3 antibodies.

The third hypervariable domain of gp120 (V3) (26) elicits potent type-specific neutralizing antibodies in immunized and experimentally infected animals (20, 30, 32). V3 antibodies block both cell-free infection and the gp120-CD4-mediated syncytium formation in vitro (25, 34). V3 antibodies do not interfere with the binding of gp120 to CD4, suggesting that the antibodies block a postbinding event required for infectivity (39). The V3 domain generates a loop structure in gp120 facilitated by an intrachain disulfide bond between the terminal cysteine residues (24). Neutralizing V3 antibodies have been shown to block a site-specific proteinase cleavage of the V3 loop in CD4-bound recombinant gp120 (7). Consequently, such antibodies have been proposed to inhibit a similar essential processing of gp120 by a host cell proteinase during the initial steps of infection (7).

The immunoglobulin G fraction of antibodies from human immunodeficiency virus (HIV)-infected chimpanzees can neutralize viral infectivity, since challenge of naive animals with virus treated with these antibodies does not lead to infection (9). Further, protective vaccination of chimpanzees with recombinant gp120 elicits high titers of V3 antibodies, whereas only low titers are found by parallel nonprotective vaccination with recombinant gp160 (2). Similar protection by vaccination has also been achieved by vaccination of chimpanzees with a mixture of inactivated virus, gp160, and V3 peptides (13). Finally, passive immunization of chimpanzees with a V3 antibody protects against infection following challenge with virus (10). Thus, these data indicate that V3 antibodies may confer part of the protection against challenge with HIV in chimpanzees. Likewise, since the majority of the HIV_{MN}-neutralizing activity in serum can be absorbed with an MN homologous V3 peptide (31) and since a neutralizing human monoclonal antibody defining an epitope in V3 has been generated from an HIV-infected individual (37), part of the neutralizing response discovered in natural infection probably also consists of V3 antibodies. However, the role of these antibodies during primary infection and their role in controlling the progression of an established HIV infection are not well characterized.

We have addressed the question of V3 antibody frequency and significance in natural infection. Using nested-primer polymerase chain reaction (PCR), we have sequenced, cloned, and expressed V3 sequences in order to determine the V3 variability and frequency of autologous antibody reactivity in HIV-infected individuals with different immunological and clinical statuses. This approach revealed that autologous antibodies against V3 were highly prevalent. Moreover, all patient sera tested in this study reacted with a vast majority of heterologous V3 proteins despite a significant amino acid variability.

MATERIALS AND METHODS

Specimen collection. Corresponding samples of serum and heparinized blood were obtained from 31 HIV antibody-positive hemophiliac patients infected with virus from different factor VIII preparations. The 31 patients represented various stages of infection with HIV (Table 1).

Separation of cells and virus culture. Peripheral blood mononuclear cells (PBMC) were isolated from HIV-infected and several healthy donors by density gradient centrifugation of heparinized blood on Lymphoprep (Nycomed, Oslo, Norway). Normal donor PBMC samples were stimulated for 3 days in RPMI 1640 (Biochrom, Berlin, Germany) supplemented with 10% (vol/vol) fetal calf serum (GIBCO, Paisley, Scotland), 10 μg of streptomycin per ml, and 100 U of penicillin (complete RPMI 1640) per ml containing 5 μg of phytohemagglutinin (PHA; Wellcome, Dartford, England)

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TABLE 1. Immunological[a] and clinical[b] statuses of the 31 HIV antibody-positive hemophiliac patients

Patient no.	PWM response (% of control)	CDC status	No. of $CD4^+$ cells/mm^3	$CD4^+$ cells (% of lymphocytes)
288	75	II/III	900	46
289	65	II/III	300	20
290	75	II/III	400	20
291	20	IV C1	0	0
292	15	II/III	200	26
293	85	II/III	600	28
294	11	II/III	200	27
295	50	II/III	400	25
296	60	II/III	400	24
297	13	II/III	300	20
298	118	II/III	700	52
299	21	II/III	500	30
300	8	IV C2	200	18
301	33	II/III	600	36
303	31	II/III	700	31
304	24	IV C2	400	21
305	16	IV C2	100	13
306	120	II/III	400	28
307	28	II/III	400	12
308	235	II/III	600	40
309	12	II/III	200	20
310	56	II/III	400	40
311	139	II/III	1,000	40
312	61	IV C2	500	32
313	90	II/III	500	38
314	56	II/III	700	23
315	21	II/III	400	16
316	43	II/III	700	31
317	59	II/III	200	33
318	73	II/III	500	36
319		II/III	600	40

[a] Normal immunological status was defined as pokeweed mitogen (PWM) response of >25% (19), $CD4^+$ count of >500 cells per mm^3 (19), and $CD4^+$ count of >29% of lymphocytes (12).

[b] As defined by the Centers for Disease Control (CDC; 6). IV C1, *Pneumocystis carinii* pneumonia; IV C2, multidermatomal herpes zoster; II, asymptomatic infection; III, persistent generalized lymphadenopathy; II/III, II or III.

per ml prior to coculture. For coculture, 5×10^6 patient PBMC were seeded at a density of 10^6 cells per ml with an equal number of PHA-stimulated donor PBMC in complete RPMI 1640 containing 10% (vol/vol) recombinant interleukin 2 (Electro Nucleonics, Silver Spring, Md.) and 2 μg of hexadimetrine bromide (Polybrene) (Aldrich Chemie, Steinheim, Germany) per ml. The cultures were maintained for 35 days, and 2×10^6 cells were exchanged weekly with an equal number of new PHA-stimulated PBMC. The cultures were screened for the presence of viral infection by reverse transcriptase assay (19) and by PCR (36).

Oligonucleotide primers. Viral DNA was cloned by nested-primer PCR amplification with the oligonucleotide outer primer set L5′ (5′AGC ACA GTA CAA TGT ACA CAT GGA AT 3′) and L3′ (5′AAA TTC CCC TCC ACA ATT AAA ACT GTG 3′) defining a DNA fragment corresponding to amino acids 249 to 389 of gp120. Amino acid numbering was as described elsewhere (33). The oligonucleotide inner primer set contained restriction enzyme recognition sequences and hybridized to the DNA sequence corresponding to amino acids 298 to 306 of gp120 in the 5′ end and to amino acids 336 to 345 of gp120 in the 3′ end. The primer set generated a DNA fragment corresponding to amino acids 298 to 345 of gp120. Thirty-three unique oligonucleotide sequences were generated from the different sequences obtained by direct sequencing of viral DNA amplified with the L5′ and L3′ oligonucleotide primers and were used as primers for the secondary amplification.

Detection and sequencing of HIV DNA. A total of 10^6 cells from cocultures were washed in phosphate-buffered saline, lysed, and deproteinated in 100 μl of 1× PCR buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 2.5 mM $MgCl_2$) containing 0.5% (vol/vol) Nonidet P-40, 0.5% (vol/vol) Tween 20, and 60 μg of freshly added proteinase K per ml, and the mixture was incubated at 55°C for 1 h and then for 10 min at 94°C to inactivate the proteinase. DNA from 10 μl of this cell lysate was amplified in a 50-μl reaction mixture with a final concentration of 1× PCR buffer, 200 μM each deoxynucleoside triphosphate (dNTP), 0.025 U of Amplitaq DNA polymerase (Perkin-Elmer, Norwalk, Conn.) per μl, and 0.4 μM each oligonucleotide primer.

PCR was performed essentially as described elsewhere (36). Briefly, DNA was amplified following 30 s of initial denaturation at 94°C by 30 cycles of denaturation for 10 s at 94°C, annealing by cooling to 55°C over 1 min and 15 s, and primer extension at 72°C for 1 s in a Thermal-Cycler (Perkin-Elmer). For each cycle, the duration of the 72°C step was extended 6 s. The amplification product was analyzed on a 1% (wt/vol) SeaKem–1.5% (wt/vol) NuSieve (FMC, Rockland, Maine) composite agarose gel stained with ethidium bromide.

DNA was further amplified for cloning. Briefly, 1 μl of product generated by amplification of DNA with the primers L5′ and L3′ was incubated in 50 μl of a mixture with a final concentration of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM each dNTP, 0.025 U of Amplitaq DNA polymerase per μl, 0.4 μM each deoxyoligonucleotide primer and amplified as described previously in this section.

Single-stranded DNA was generated by asymmetric PCR (17) and used for direct sequencing of the primary amplification product. Single-stranded DNA and double-stranded plasmid DNA were sequenced with Sequenase (U.S. Biochemical, Cleveland, Ohio) according to the instructions of the manufacturer.

Production and partial purification of recombinant protein. PCR-generated DNA fragments were digested with the restriction enzymes *Eco*RI and *Bam*HI and ligated into the *Escherichia coli* expression vector pUEX (4). Recombinant plasmids were transformed into the *E. coli* strain NF3082 (41), and following plasmid amplification, the sequences of the recombinant plasmids were verified by sequencing. Bacteria were grown in NY medium (0.8% [wt/vol] NZ-amine, 0.5% [wt/vol] yeast extract [Difco, Detroit, Mich.], 0.5% [wt/vol] NaCl) buffered with 17 mM KH_2PO_4 and 70 mM K_2HPO_4.

For induction of plasmid-encoded recombinant fusion protein, bacterial cultures were grown at 30°C to an optical density at 600 nm of 0.2. The temperature was elevated to 42°C, and growth was maintained for an additional 1 h and 40 min before the bacteria were harvested and cooled on ice. Recombinant fusion protein was partially purified from large-scale bacterial cultures as described previously (8). Protein concentrations were determined by a colorimetric protein assay (Bio-Rad, Richmond, Calif.).

Western blot. Partially purified fusion protein was mixed with an equal volume of 2× sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis sample buffer containing 5% (wt/vol) SDS and was heated briefly to 100°C. A sample of 10 μl was loaded onto a 7.5% polyacrylamide gel, and proteins were separated by electrophoresis. Separated pro-

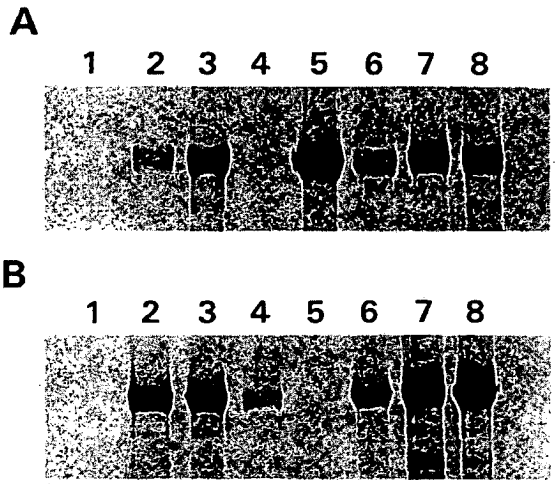

FIG. 1. Western blot detection of V3 antibodies in HIV antibody-positive patient sera. (A) Proteins detected with serum sample no. 290. Lanes (score given in parentheses): 1, parental Cro-β-galactosidase fusion protein (−); 2, protein no. 289 (1); 3, protein no. 290 (3); 4, protein no. 291 (−); 5, protein no. 294 (3); 6, protein no. 295 (2); 7, protein no. 311 (3); 8, protein no. 313 (3). (B) Proteins detected with serum sample no. 289. Lanes (score given in parentheses): 1, parental Cro-β-galactosidase fusion protein (−); 2, protein no. 303-A (3); 3, protein no. 303-B (3); 4, protein no. 304 (1); 5, protein no. 305 (between − and 1); 6, protein no. 306-5 (3); 7, protein no. 306-7 (3); 8, protein no. 310 (3).

teins were transferred electrophoretically to 0.45-μm-pore-size polyvinylidene membranes (Millipore, Bedford, Mass.), and the membranes were blocked with 2% (vol/vol) Tween 20 in phosphate-buffered saline and then incubated with patient serum diluted 1:200 in washing buffer (50 mM Tris-base [pH 10.2], 150 mM NaCl, 0.1% [vol/vol] Tween 20) containing 20% (vol/vol) *E. coli* lysate, 1% (wt/vol) bovine serum albumin, 1% (vol/vol) normal rabbit serum (Dakopatts, Glostrup, Denmark), and 5 mM NaN_3. Patient antibodies were detected with peroxidase-conjugated rabbit anti-human immunoglobulin G antibody (Dakopatts) by the dioctyl sodium sulfosuccinate-tetramethylbenzidine staining method (22). The results were arbitrarily scored according to the following four categories on the basis of visual evaluation of the staining intensity: −, no reactivity; 1, weak reactivity; 2, moderate reactivity; 3, good reactivity (Fig. 1).

Amino acid sequence accession number. The amino acid sequences listed in Table 2 are available under EMBL data library accession numbers P80112 through P80142 and P04578.

RESULTS

Culture of virus and amplification of the corresponding V3 DNA. To ensure that V3 sequences were amplified from replication-competent HIV, virus was propagated from patient PBMC by coculture with PHA-stimulated normal donor PBMC. PBMC from hemophiliac patients infected with HIV from different factor VIII preparations were used for virus isolation to ensure diversity of the viral strains. To overcome the potential problems of primer mismatch that may arise when PCR is applied for amplification of hypervariable nucleotide sequences, viral DNA was amplified by nested-primer PCR. Cell cultures were screened for HIV infection with an outer primer pair which was complementary to conserved *env* regions and produced a DNA fragment corresponding to amino acids 249 to 389 of gp120. Subsequently, the viral DNA was sequenced, and inner oligonucleotide primers matching the individual DNA fragments perfectly were generated for amplification and cloning of the DNA corresponding to V3. The efficiency of the screening with the outer primer pair was initially monitored by double screening of 10 cultures by reverse transcriptase assay and PCR. The results of the two screening methods were identical (data not shown), since a DNA fragment containing V3 could be amplified from all reverse transcriptase-positive cultures with the primers L5′ and L3′. Moreover, the amplification strategy excluded the risk of primer-induced mutagenesis of V3 by the use of perfect matching primers for generation of the secondary PCR product.

Virus was isolated from 24 patient samples, and viral DNA was subsequently amplified. Viral DNA was amplified from 24 cultures at day 9 after infection, and the remaining 7 cultures were further maintained until day 35; however, no viral DNA could be amplified from these cultures despite attempts at several time points. In addition, viral DNA was amplified from a PBMC culture infected with the reference isolate human T-cell leukemia virus IIIB (HTLV-IIIB). Direct sequencing of the amplified DNA fragments (each containing the entire nucleotide sequence of V3) showed that all sequences were unique. The products of at least three individual PCR amplifications from each culture were directly sequenced and compared to detect any possible *Taq* polymerase-induced point mutations; however, none was observed.

None of the 25 DNA fragments contained stop codons or frameshift mutations in the region corresponding to V3, but the DNA amplified from eight patients contained a mixture of two nucleotides at various positions on the sequence ladder (data not shown). Moreover, the sequence amplified from patient no. 291 and the HTLV-IIIB-infected cell sequence contained insertions of 12 and 3 nucleotides, respectively, compared with the remaining sequences (data not shown). The sequence of the V3 DNA amplified from HTLV-IIIB-infected cells was identical to that of the molecular clone HXB2.

Cloning and expression of amplified DNA fragments. The primary amplification product corresponding to amino acids 249 to 389 was used as the template for a second amplification with oligonucleotide primers, producing a DNA fragment corresponding to amino acids 298 to 345. These secondary PCR products were cloned into the prokaryotic expression vector pUEX, and the sequences of the constructions were verified by sequencing. Each of the cloned V3 sequences (Table 2) corresponded perfectly with the sequences obtained by direct sequencing of the primary PCR products.

From five of the primary amplification products derived from patients no. 288, 296, 303, 306, and 314, which contained mixed genotype in some nucleotide positions, more than one clone was selected. The specific sequences of these additional clones corresponded with the locations of the mixed nucleotides in the primary amplification products, indicating that the mixed nucleotides represented true heterogeneity in the viral strain. Thus, from the 25 primary amplification products, 32 different DNA fragments corresponding to amino acids 298 to 345 of gp120 were cloned into pUEX, including the HXB2 V3 fragment (Table 2). Sequence analysis showed that the variability within the V3 domain was confined to certain clusters of the sequence and that apart from the terminal cysteine residues, the central

TABLE 2. Maximum alignment of cloned V3 sequences corresponding to amino acid residues 298 to 345 of gp120 from hemophiliac patients[a]

Consensus or construct[b]	Sequence[c]
Consensus	S V E I N C T[d] R P N N N T R K S I H I . . . . G P G R A F Y T T G D I I G D I R Q A H C N L S R A K W
Group 1[e]	
288-1	– – – – – – – – – – – – – – – – – – N – . . . . –
288-3	P – – – – – – – – – – – – – – – – – – N – . . . . –
288-4	P – – – – – – – – – – – – – – – – – – N – . . . . – – – – – – – – – – – A – – – – – – – – – – – – – I – E –
289	P – – – – – – – – S – – – – R – – N – . . . . – – – – – – – – – – – E V – – N – – – – – – – – I – G T P –
290	– – A – – – – – – – – – – – – – – – – . . . . – – – – – – – – A – – E – – – – – – – – – – – V – – T – –
292	– – K – – – – – – – – – – – – – – G – – – . . . . – – – – – – – – – – – Q – – – – – – – – – – – – E E –
294	P – D – – – – – – – – – – – – – – – – – . . . . – – – – – – – – A – – – – – – – – – – – – – – I – I T L –
295	– – A – – – – – – – – – – – – – – – N – . . . . – – – – – – – – – – – E – – – – – – – – – – – – I – – – Q –
296-A	– – V – – – – – – H – – – – – G – – – . . . . – – – – – – – – A – – – – – – – – – – – – – – – – G – – –
296-E	– – V – – – – – – H – – – – – G – R – . . . . – – – – – – – – A – E R – – – – – – – – – – – – – G – – –
297	P – – – – – – – – S – – – – – – – N – . . . . – – – – – – – – – – – A – – – – – – – – – – – – – M – L –
299	– – – – – – – – – – – – – – – – – G – – – . . . . – – – – – – – – – – – – – – – – N – – – – – – – – – D –
300	T – D – – – – – – – – – – – – – – G – – – . . . . – – – – – – – – A – – – – – – – – – – – – – I – – – – –
301	– – T – – – – – – – – – – – – – – – – – . . . . A – – – – – – – A – – – – V – – – – – – – – – I – K – E –
303-A	P – – – – – – – – – – – – – – – – – – – . . . . – T – –
303-B	T – – – – – – – – – – – – – – – – – – – . . . . – T – –
306-5	– – – – – – – – – H – – – – – – – S – – . . . . – – – – – – L – A – – G – – – – – – – – – – – – – – – –
306-7	– – – – – – – – – H – – – – – – – Y – – . . . . – – – – – – W – A – – E – T – – – – – – – – – – – – – –
310	P – K – – – – – – G – – – – – G – – – . . . . – – – – – – – – – – – S – T – – – – – – – – – I – – K D –
311	– . . . . –
313	– I – – – – – – – S – – – – – – – – – – . . . . – – – – – – – – – – – E – T – – – – – – – – – I – – – –
314-B	– – Q – D – I – – – – – – – – – – – – – . . . . A – K – – –
314-C	– – K – D – I – – – – – – – – – – – – – . . . . A – K – – –
315	– . . . . – – – – – – – – – – – E – – – – – – – – – – – – – – – –
316	– . . . . – – – – – – – – – – – E – – – – – – – – – – – – – – E –
319	– – D – T – I – – H – – – – – G – – – . . . . – – – – – – – – A – – – – – – N – – – – – – – I – – – D –
Group 2	
288-2	– – – – – – – – – – – – – – – – R G – – M . . . . – – – – G – – – – – – – E – – – – – – – – – – – – I – E –
305	– – – – – – – – – – S – – – – – – – – L . . . . – – – – G – – – – A – – A – – – – – – – – – – – – – – D –
317	– – – – – – – – – – – – – – – – – S – – – – . . . . – – – – G – – R – – – R – – – – – – – – – – – – T N S –
Group 3	
304	– – – – – – – – – – – – – – – – – R – – P – . . . . – – – – – – – – A – – – – – – – – – – – – Y – – I – K – – –
Group 4	
291	– – – – – – – – – – – – – – – – – G – R – G T G I – L – S T – – A – E L – – – – – – – – – – – – – – – –
Group 5	
HXB2	– – – N – – – – – – – – – – – – R – R – Q R . . – – – – – – – V – I – K – . – N M – – – – – – I – – – – –

[a] Amino acid numbering as described elsewhere (33).
[b] Three-digit numbers denote the patient origin of each construct.
[c] —, homology with consensus; -, gaps introduced to obtain maximum alignment.
[d] NXS and NXT N-linked glycosylation sites. X can be any amino acid other than proline.
[e] Groupings based on related serological reactivities.

sequence GPGRAF and the C-terminal sequence RQAH were well conserved among the majority of the V3 fragments. Although the GPGRAF central sequence was not conserved among all of the constructions, a potential cleavage site for thrombin-, tryptase-, and cathepsin E-like proteinases was present in the central motif of all cloned V3 sequences. Likewise, the amino acids constituting three potential asparagine-linked glycosylation sites were conserved in all of the V3 sequences, except sequences no. 314-B, 314-C, and 319, in which only two glycosylation sites were conserved (Table 2).

Reactivity of patient sera in Western blots. To analyze the autologous antibody response, recombinant V3 fusion protein was produced in *E. coli* harboring recombinant pUEX plasmids. The bacteria produced large amounts of Cro-β-galactosidase-V3 fusion protein from all of the plasmid constructions. Comparison of the electrophoretic mobilities of the recombinant and parental plasmid-encoded fusion proteins confirmed that the recombinant proteins had the expected molecular weights (data not shown).

Initially, all recombinant fusion proteins were tested with a pool of HIV antibody-negative sera ($n = 50$) without detection of reactivity (data not shown). Conversely, a pool of HIV antibody-positive sera ($n = 50$) reacted with the recombinant fusion proteins but did not react with the parental Cro-β-galactosidase fusion protein produced by induction of the native pUEX vector (data not shown).

Equal amounts of recombinant bacterial fusion protein were tested with the patient sera. Twenty-four serum samples corresponding to the PCR-positive patient samples were tested for reactivity with the 31 autologous recombinant fusion proteins (Tables 2 and 3). Further, serum samples from all 31 patients (the 24 PCR-positive patients and the 7 PCR-negative patients from whom viral DNA could not be amplified) were cross-tested for antibody reactivity against all 32 heterologous recombinant fusion proteins (Table 4).

All sera tested in this study contained autologous antibody response against the corresponding antigen, with the exception of serum sample no. 291. A similar lack of reactivity with protein no. 291 was also found for four consecutive autologous serum samples dating 3 years back and for a serum pool derived from 50 HIV-positive individuals (data not shown). However, serum sample no. 291 readily reacted with the majority of the heterologous V3 fusion proteins (Table 4), and V3 antibodies were thus found in all of the serum samples, independently of the immunological and clinical statuses of each patient (Table 1). Most proteins containing the central sequence PGRAFYTT or PGRAFY AT reacted with heterologous serum samples from patients with an identical sequence. In contrast, the HXB2 V3 sequence reacted moderately with only one serum sample, whereas five samples reacted weakly. Moreover, protein no. 304 containing a proline residue adjacent to the central sequence (Table 2) reacted with a small number of heterologous serum samples. Finally, proteins no. 288-2, 305, and 317, each containing an R→G substitution in the central sequence, reacted with a small number of heterologous serum samples, whereas substitutions in other positions of this sequence (proteins no. 301, 306-5, 306-7, 314-B, and 314-C) affected the reactivity with heterologous sera to a lesser extent.

Accordingly, amino acid insertions and some substitutions within the central V3 sequence PGRAFYTT appeared to affect the reactivities of heterologous sera against the recombinant proteins. Thus, proteins no. 288-2, 291, 304, 305, and 317 and the HXB2 V3 protein reacted with a smaller number of serum samples than did the remaining proteins (Tables 2 and 3).

TABLE 3. Western blot reactivity of all serum samples with the individual recombinant fusion proteins[a]

Protein no. or name	Autologous reactivity[b]	No. of serum samples with heterologous reactivity[c]			
		–	1	2	3
Group 1[d]					
288-1	2	2	7	18	3
288-3	3	1	3	6	20
288-4	3	0	3	7	20
289	3	1	4	4	21
290	3	0	2	3	25
292	2	0	13	14	3
294	3	0	0	0	30
295	1	1	2	4	23
296-A	3	0	1	7	22
296-E	3	4	6	6	14
297	3	0	3	5	22
299	3	0	1	5	24
300	3	0	1	4	25
301	3	2	3	11	14
303-A	3	0	1	2	27
303-B	3	0	1	3	26
306-5	3	2	8	10	10
306-7	3	2	4	5	19
310	3	0	0	1	29
311	3	1	0	1	28
313	3	0	1	2	27
314-B	3	0	7	14	9
314-C	3	0	6	15	9
315	3	0	1	1	28
316	3	0	0	2	28
319	3	0	1	5	24
Group 2					
288-2	–	18	8	4	0
305	3	18	7	3	1
317	1	7	8	11	4
Group 3					
304	3	7	10	9	4
Group 4					
291	–	30	0	0	0
Group 5					
HXB2	ND	25	5	1	0

[a] The V3 amino acid sequences of the proteins are listed in Table 2.
[b] Graduated reactivity of patient sera with the autologous recombinant fusion proteins: 3, good; 2, moderate; 1, weak; –, no reactivity; ND, no data (Fig. 1).
[c] Number of graduated reactivities of heterologous patient sera with the individual recombinant fusion proteins. See footnote *b* for description of scoring code.
[d] Groupings based on related serological reactivities.

DISCUSSION

Despite the extensive humoral and cellular immune response following challenge with HIV, the infection persists and appears to evade the immune system. The virus envelope proteins are major targets for antibody responses, and several B-cell epitopes have been mapped elsewhere in detail (5, 14–16, 21, 29, 30, 37, 40), but only epitopes in V3 appear to elicit high titers of in vitro neutralizing antibodies (3, 16, 37).

TABLE 4. Western blot reactivity of individual patient serum samples with all recombinant fusion proteins[a]

Serum no.[b]	No. of recombinant fusion proteins with the following degrees of reactivity[c]:			
	–	1	2	3
Group 1[d]				
288	3	5	3	21
289	5	6	7	14
290	4	11	4	13
291	4	3	10	15
292	4	2	3	23
293[e]	5	5	11	11
294	3	1	4	24
295	6	6	9	11
296	1	5	7	19
297	2	3	3	24
298[e]	8	9	9	6
299	3	2	6	21
300	5	0	2	25
301	6	3	4	19
303	2	0	6	24
304	3	4	8	17
306	4	5	1	22
307[e]	4	9	12	7
308[e]	5	6	8	13
309[e]	5	5	8	14
310	2	3	4	23
311	3	4	1	24
312[e]	2	2	5	23
313	4	1	6	21
314	4	1	3	24
315	4	1	7	20
316	1	1	8	22
317	4	3	7	18
318[e]	4	1	7	20
319	3	0	7	22
Group 2				
305	11	12	5	4

[a] The antibody reactivity of each serum sample was tested against 32 recombinant fusion proteins.
[b] Numbers denote the patient origin of each serum sample.
[c] Reactivities of patient sera with the recombinant fusion proteins were scored as follows: 3, good; 2, moderate; 1, weak; –, no reactivity (Fig. 1).
[d] Groupings based on related serological reactivities.
[e] Serum sample not tested against autologous fusion protein.

In the present study, we chose to isolate viruses by cocultivation on PBMC. This ensures that only V3 sequences originating from replication-competent virus are analyzed but may also impose a bias on the study, since a substantial selection of nonrepresentative viral populations cannot be excluded. Alternatively, V3 sequences could have been obtained by cloning from serum samples by reverse transcription of viral genomic RNA and subsequent PCR. However, this method suffers from the drawback that V3 sequences from replication-incompetent viral genomes can be isolated.

The sequence variability of V3 among patients and the strain variability within individual patients demonstrated here are in agreement with previous observations (11). However, this is the first study of a larger series of patients which clearly demonstrates that autologous V3 antibody responses are almost ubiquitously present in patients independently of their clinical and immunological statuses. Further, previous observations of type specificity of V3 antibodies in immunized animals (20) indicate that the amino acid variability found in V3 (27) may create an indefinite number of serotypes. However, our study tends to suggest that the variability of the V3 antigenic determinants in field isolates is less extensive than the variability among molecular clones of HIV from different laboratories (27).

By cross-testing of sera and recombinant fusion proteins, the amino acids of particular importance for antibody reactivity appeared to reside within the central sequence PGRAFYTT. This sequence has previously been shown to constitute an epitope defined by a human monoclonal antibody (37). Fusion proteins containing a central PGRAFYTT or PGRAFYAT sequence reacted with the majority of heterologous sera, whereas heterologous reactivity against proteins no. 288-2 (GIHMGPGGAFYTT), 305 (SIHLGPG GAFYAT), and 317 (SIHIGPGGAFRTT) (Table 3) was detected only infrequently. Accordingly, serum sample no. 305 reacted with a small number of proteins, but proteins no. 305, 288-2, and 317, which harbored the PGGAFYTT type of central sequence, were well recognized. Thus, serum sample no. 305 appeared to define a serotype characterized by the infrequent substitution of PGRAFYAT to PGGAFYAT, PGGAFRTT, or PGGAFYTT. A similarly infrequent antibody reactivity against the HXB2 V3 protein (RIRI[QR]GP GRAFVTI) was detected, whereas no antibody reactivity against protein no. 291 (GIRI[GTGI]GLGSTFYAT) in any sample (including autologous samples dating 3 years back) was detected.

Insertions such as those found in protein no. 291 and the HXB2 V3 protein may have caused the significant effect on antibody recognition, probably by altering the structure of the putative epitope. Moreover, the central sequence of both proteins contained amino acid substitutions compared with the consensus sequence PGRAFYTT.

Further, reduced reactivity was observed against protein no. 304 (SIPIGPGRAFYAT) containing an additional proline residue proximal to the central sequence. The location adjacent to the site of a predicted β turn in the V3 loop (23) probably affects the structure of the loop and prevents antibodies in heterologous sera from binding despite conservation of the remaining sequence PGRAFYAT. In addition, amino acid substitutions in the center of V3 were observed in proteins no. 301, 306-5, 306-7, 314-B, and 314-C. However, these substitutions did not appear to affect the reactivities of heterologous antibodies to any greater extent.

Thus, amino acid substitutions within the PGRAFYTT sequence in positions 1, 2, 3, and 4 and to some extent position 5 appeared to have the most prominent effect on heterologous reactivity, whereas substitutions in positions 6, 7, and 8 had less effect. In addition, the presence of a proline residue adjacent to the central sequence as found in protein no. 304 had a marked effect on heterologous antibody reactivity, as previously described (42). Accordingly, less-frequent antibody reactivity was detected in heterologous sera against fusion proteins containing insertions and/or an aberrant amino acid composition in the central part of the V3 sequence.

Since parts of V3 are conserved and since the major antigenic determinants appear to reside only in the center of V3, the number of serotypes appears to be small. However, the insertions in protein no. 291 and the HXB2 V3 protein indicate that viral escape of immune surveillance ultimately leads to strongly aberrant V3 types. Thus, more-drastic modifications of V3 are tolerated, provided that functional properties of gp120 are retained. The number of serotypes may thereby be expanded in response to selective pressure, but such strongly aberrant types appear to occur only

infrequently (23) and are not found in experimentally infected chimpanzees (28).

During primary infection of humans and chimpanzees, the neutralizing effect of antibodies against concurrently isolated virus declines (1, 28). Analysis of V3 from chimpanzees reveals that mutations in V3 appear subsequent to the onset of the stage of neutralization escape. Accordingly, neutralization-resistant viruses isolated early do not contain altered V3 sequences, whereas prechallenge vaccination with V3 peptides generates a rapid antigenic drift in V3 and accelerates neutralization escape (28). These observations may explain our frequent detection of autologous V3 antibodies in all stages of the infection. Thus, despite a potent in vitro neutralizing effect, V3 antibodies may apparently mediate only an early and transient selective pressure on the virus in vivo (28). Ultimately, this may be unable to prevent progression of the latent infection into significant immunodeficiency, although our present data cannot fully support this assumption since we address only the frequency of V3 antibody binding.

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